(12) **EUROPEAN PATENT SPECIFICATION**

(54) **Humanized and chimeric monoclonal antibodies that recognize epidermal growth factor receptor (EGF-R); diagnostic and therapeutic use**

Humanisierte und chimäre monoklonale Antikörper die den Rezeptor für epidermalen Wachstumfaktor (EGF-R) erkennen; diagnostische und therapeutische Verwendung

Anticorps monoclonaux humanisés et chimériques que reconnaissent le récepteur du facteur de croissance épidermique (EGF-R); utilisation diagnostique et thérapeutique

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Description

FIELD OF THE INVENTION

5   [0001]   This invention is related to the field of immunology and in particular to two new products, which are chimeric and humanized monoclonal antibodies against EGF-R, less immunogenic than the original murine monoclonal antibody and with improved effector functions.

[0002]   This invention also relates to therapeutic and diagnostic compositions comprising these antibodies.

10   BACKGROUND OF THE INVENTION

[0003]   The Epidermal Growth Factor (EGF), which is a 53 amino acid polypeptide with a molecular weight of 6045 D, was isolated and purified for the first time from murine submaxillary gland (Cohen S; J Biol Chem (1962) 237, 1555). Later, a similar molecule was obtained from human urine (Cohen S and Carpenter G; (1975) PNAS USA 72, 1317).
15   The action of this polypeptide is mainly performed via its membrane receptor, a 170 KD molecular weight glycoprotein. Its intracellular domain is associated with a tyrosine kinase activity with structural homology to the oncogene v-erb-B showing relation to the malignant transformation process (Heldin CH et al; (1984) Cell 37, 9-20).

[0004]   High levels of EGF-R have been detected in malignant tumors originating in the epithelium such as breast, bladder, ovarian, vulva, colonic, lung, brain and esophagus cancers. The role played by EGF and its receptor in reg-
20   ulating tumor growth is unknown, but it has been suggested that the EGF-R expression in tumor cells provides a mechanism for autocrine growth stimulation which leads to uncontrolled proliferation (Schlessinger J, Schreiber AB, Levi A, Liberman T and Yarden Y; (1983) Crit Rev Biochem 14(2), 93-111).

[0005]   It has been reported that EGF produces overgrowth of breast cancer cell lines (Osborne CK et al; (1980) Cancer Research 40, 2361), besides that it modulates the differentiation under some cellular systems (Tonelli CJ;
25   Nature (1980) 285, 250-252). These effects on cellular differentiation and proliferation are related to the high expression of EGF-R (Buss JE et al; (1982) PNAS 79, 2574).

[0006]   The presence of EGF-R in tumor cells has proven to be an indication of a poor prognosis in human breast cancer. Approximately 40% of the breast tumors show specific binding sites of high affinity for the EGF (Perez R, Pascual MR, Macias A, Lage A; (1984) Breast Cancer Research and Treat 4, 189-193). There is also an inverse
30   correlation with the presence of estrogen receptor, indicating EGF-R as an indifferentiation marker or an indicator of the potential capacity of proliferation of the malignant cells.

[0007]   Other groups have reported that the expression of EGF-R is higher in regional ganglionar metastasis than in primary carcinomas of breast (Sainsbury JR et al; (1985) Lancet 1, 8425, 364-366) and that the expression of the receptor is different in the different histologic subtypes of human breast carcinoma cells, their presence constituting a
35   signal of bad prognosis (Macias A et al; Anti Cancer Research 6: 849-852).

[0008]   The evidences obtained in different studies have prompted to consider the EGF/EGF-R system as a possible target for therapeutic actions.

[0009]   We obtained a murine monoclonal antibody (R3), raised against the human placenta as described in European Patent publication No. EP-A-0 586 002, and found to bind to the external domain of the human EGF-R. It was found
40   to inhibit the binding of EGF at both low and high affinity EGF-R sites.

[0010]   Passive immunotherapy using monoclonal antibodies against the EGF-R have been the object of multiple investigations, which have demonstrated that the specific recognition of the receptor by the antibody inhibits the EGF binding, with an inhibitory effect on the mitogenic stimulation of malignant cells (Sato JD et al; (1987) Methods in Enzimology 148, 63-81); but there are evidences that the murine origin of these antibodies produces a human anti-
45   mouse antibody response.

[0011]   The development of the hybridoma antibody technique by Kohler and Milstein revolutionized the discipline of immuno-chemistry and provided a new family of reagents with potential applications in clinical diagnosis and immunotherapy (Kohler G, Milstein C; (1975) Nature 256, 495-497). While it has become routine to produce mouse monoclonal antibodies (mAbs) for use in basic research and clinical diagnosis, it has been difficult to use these for in vivo
50   immunotherapy because they have reduced half life in humans, poor recognition of mouse antibodies effector domains by the human immune system and the foreign immunoglobulin can elicit an antiglobulin response (HAMA response) that may interfere with therapy.

[0012]   The ability to genetically manipulate antibody genes and then to express these altered genes by transfection techniques enables us to produce mAbs having more desirable properties than the existing hybridoma antibodies.
55   Thus genetic engineering can be used to enhance desired effector functions in antibody molecules and to decrease or eliminate undesired effector functions.

[0013]   The use of recombinant DNA technology to clone antibody genes has provided an alternative whereby a murine mAb can be converted to a predominantly human form with the same antigen binding properties. SL Morrison

in 1984 created mouse-human antibody molecules of defined antigen-binding specificity by taking the variable regions genes of mouse antibody producing myeloma cell lines and joining them to human immunoglobulin constant region (Morrison SL et al; (1984) PNAS USA 81, 6851-6855).

[0014] Other authors have attempted to build rodent antigen binding sites directly into human antibodies by transplanting only the antigen binding site, rather than the entire variable domain, from a murine antibody (Jones PT et al; (1986) Nature 321, 522-524; Verhoeven M et al; (1988) Science 239, 1534-1536). Some applications of this method have been developed (Rietchmann L et al; (1988) Nature 332, 323-327; Quee C et al; (1989) PNAS USA 86, 10029-10033), other authors have worked with reshaped antibodies, which included some murine residues in human FRs in order to recover the affinity for the original antigen (Tempest PR; (1991) Biotechnology 9, 266-272).

[0015] Orlandi R et al (Proc Natl Acad Sci USA 86, 3833-3837, 1989) disclose the constant regions of the human gamma-1 heavy chain and the human kappa light chain, and suitable cloning vectors therefor.

[0016] Thus, an object of the invention is to provide a chimeric and a humanized mAb which are, in particular, directed to the EGF-R, comprising an antigen-binding site of non-human sources and the constant regions of human origins (chimeric) and the FRs of the variable regions and constant regions of human origins, which are, if necessary, modified in a way that the specificity of the binding can be conserved or restored.

[0017] In particular, an object of the invention is to characterize the hypervariable regions of the antigen-binding site of an antibody against the EGF-R and providing these CDRs within a humanized and chimeric mAb defined as above.

[0018] These antibodies can play a role as a therapeutic or diagnostic agent in order to combat tumors with high expression of EGF-R.

SUMMARY OF THE INVENTION

[0019] This invention provides a monoclonal antibody which specifically binds epidermal growth factor receptor (EGF-R), characterized in that it is a chimeric or a humanized antibody which contains the following complementarity determining regions (CDRs):

CDR1 of the light chain: **RSSQNIVHSNGNTYLD,**
CDR2 of the light chain: **KVSNRFS,**
CDR3 of the light chain: **FQYSHVPWT,**
CDR1 of the heavy chain: **NYYIY,**
CDR2 of the heavy chain: **GINPTSGGSNFNEKFKT,**
CDR3 of the heavy chain: **QGLWFDSDGRGFDF.**

[0020] Monoclonal antibodies of this invention bind to human EGF-R and are capable of inhibiting the binding of EGF to EGF-R.

[0021] In a preferred embodiment, said antibody is a chimeric antibody having the following variable regions of the light and heavy chains:

variable region of the light chain:

DVLMTQIPLSLPVSLGDQASISCRSSQNIVHSNGNTYLDWYLQKPGQSPNL
LIYKVSNRFSGVPDRFRGSGSGTDFTLKISRVEAEDLGVYYCFQYSHVPWT
FGGGTKLEIKRA;

variable region of the heavy chain:

QVQLQQPGAELVKPGASVKLSCKASGYTFTNYYIYWVKQRPGQGLEWIGGI
NPTSGGSNFNEKFKTKATLTVDESSTTAYMQLSSLTSEDSAVYYCTRQGL
WFDSDGRGFDFWGQGTTLTVSS.

[0022] In another preferred embodiment, said antibody is a humanized antibody having the following variable regions of the light and heavy chains:

variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKL
LIYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTF
GQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGI
NPTSGGSNFNEKFKTx^1x^2TITVDESx^3x^4TAYMELSSLRSEDTAFYFCx^5RQGL
WFDSDGRGFDFWGQGSTVTVSS,

wherein x^1 is R or K; x^2 is V or A; x^3 is T or S; x^4 is N or T; and x^5 is A or T.

[0023] More particularly, preferred embodiments of said humanized antibody have the following variable regions of the light and heavy chains:

variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKL
LIYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTF
GQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGI
NPTSGGSNFNEKFKTRVTITVDESSTTAYMELSSLRSEDTAFYFCTRQGLW
FDSDGRGFDFWGQGSTVTVSS;

or
variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKL
LIYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTF
GQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGI
NPTSGGSNFNEKFKTKATITVDESSTTAYMELSSLRSEDTAFYFCTRQGLW
FDSDGRGFDFWGQGSTVTVSS;

or
variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKL
LIYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTF
GQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGI
NPTSGGSNFNEKFKTRVTITVDESSTTAYMELSSLRSEDTAFYFCARQGLW
FDSDGRGFDFWGQGSTVTVSS;

or
variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKL
LIYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTF
GQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGI
NPTSGGSNFNEKFKTRVTITVDESTNTAYMELSSLRSEDTAFYFCTRQGL
WFDSDGRGFDFWGQGSTVTVSS;

or
variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKL
LIYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTF
GQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGI
NPTSGGSNFNEKFKTKATITVDESTNTAYMELSSLRSEDTAFYFCTRQGL
WFDSDGRGFDFWGQGSTVTVSS.

[0024]   It is preferred that the constant regions of the heavy chain of said antibody comprise the amino acid sequence of a gamma-1 chain of a human immunoglobulin and the constant regions of the light chain of said antibody comprise the amino acid sequence of a kappa chain of a human immunoglobulin.

[0025]   A particularly preferred embodiment of the invention is the antibody produced by the cell line hr-R3, ECACC 951110101.

[0026]   This invention also provides said cell line hr-R3, ECACC 951110101.

[0027]   Furthermore, the invention provides a pharmaceutical composition comprising a monoclonal antibody as defined herein and a pharmaceutically acceptable carrier, in particular such pharmaceutical composition for use against tumors.

[0028]   Further, the invention provides a diagnostic reagent for localization and assessment of growth of a tumor,

comprising a monoclonal antibody as defined herein.

BRIEF DESCRIPTION OF THE FIGURES

[0029]

FIGURE 1: Deduced aminoacid sequence of (a) VK and (b) VH of murine R3 antibody. CDRs are underlined.

FIGURE 2: Aminoacid sequences of murine and humanized VK(a) and VH(b) containing mAb R3 CDRs.

FIGURE 3: Detection of binding of the chimaeric and humanized R3 to EGF-R by RRA. Antigen binding activity was assayed in different concentrations of purified murine R3 (+), chimaeric R3 (*) and humanized (1) R3 (-.-) and plotted as CPM of bound ^{125}I-EGF against log of the concentration of each antibody. (concentration of IgG was quantitated by ELISA, see detailed description of the invention).

FIGURE 4: Detection of binding of hybrids murine R3 VH/ humanized R3 VK and humanized R3 VH/murine R3 VK. The antigen binding activity was assayed in dilutions concentrated supernatants from transfected NSO cells and plotted as cpm (membrane bound radioactivity) against log of the concentration of IgG estimated by ELISA, see experimental protocols.

FIGURE 5: Comparison of the aminoacid sequences of reshaped humanized R3 heavy chain variable region. The CDRs are underlined.

FIGURE 6: Molecular Model of the Variable region of murine mAb R3. The binding site is at the top and the molecule has been slightly rotated clockwise around a vertical axis so that VH domain approaches the viewer. The VL framework is light blue and the VH framework is pink. The corresponding CDRs are marked in dark blue and red. Side chains of residue Ser 75, Thr 76 and Thr 93 (with polar hydrogen atoms included) are shown in green. The side chain of the Phe 100f is shown in red.

FIGURE 7: Detection of binding of the different reshaped mAb R3 to EGF-R by RRA. Antigen binding activity was assayed in dilutions concentrated supernatants from transfected NSO cells and plotted as cpm (membrane bound radioactivity) against log of the concentration of IgG estimated by ELISA, see experimental protocols. All versions of reshaped human VH regions were cotransfected with huVKR3 and are represented in the figure.

FIGURE 8: Immunogenicity in monkeys of R3 murine mAb and different recombinant versions. Cercopithecus aethiops monkeys were immunized intradermically at 15 days intervals with 2 mg of the different mAbs versions using aluminium hydroxide as adjuvant. The detection of monkey IgG against the different versions of R3 mAb was tested in the sera of the animals using an indirect ELISA.

DETAILED DESCRIPTION OF THE INVENTION

cDNA Synthesis and Gene Amplification of variable region of R3

[0030]   Cytoplasmic RNA was extracted from about 10^6 hybridoma cells of R3 (IgG 2A), this antibody was obtained by us (Fernandez A et al; (1989) IFN y Biotecnologia 6(3), 289-298). The method used to extract RNA was described by Faloro et al (Faloro J, Treisman R and Kemen R; (1989) Methods in Enzymology 65: 718-749).

[0031]   The cDNA synthesis reaction mixture was done as described Tempest et al (Tempest PR, Bremner P, Lambert M, Taylor G, Furze JM, Carr FDJ and Harris WJ; (1991) Biotechnology 9: 266-271). Briefly, a 50ul reaction mixture containing 5 ug of mRNA, 25 pmol of Vh or VK primer FOR, 250 uM of each dNTP, 10 mM DTT, 50 mM Tris-HCl (pH 8.3), 8 mM MgCl$_2$, 75 mM KCl and 15 units of RNAse inhibitor, was heated at 70°C, for 10 min and slowly cooled to 37°C over a period of 30 min. Then, 100 units MLV reverse transcriptase (BRL) were added and the incubation at 37°C continued for 1 hour.

[0032]   The VH and VK cDNAs were amplified using the PCR as described by Orlandi et al (Orlandi R, Gussow DH, Jones PT and Winter G; (1989) Proc Natl Acad Sci USA 86: 3833-3837). For PCR amplification of VH, DNA/primers mixture consisted of 5 ul cDNA, 25 pmoles CG2AFOR and VH1BACK primers. For PCR amplification of VK, DNA/ primers mixture consisted of 5 ul cDNA, 25 pmoles CK2FOR and VK10BACK primers. To these mixtures were added 2.5mM of each dNTP, 5 ul constituents of 10X buffer thermolase and 1 unit of Thermolase (IBI) in a final volume of 50 ul. Samples were subjected to 25 thermal cycles at 94°C, 30sec; 50°C, 30sec; 72°C, 1 min; and a last incubation for 5 min at 72°C. Amplified VH and VK DNA were purified on Prep A Gene purification kit (BioRad).

Cloning and Sequencing of Amplified cDNA

[0033]   The purified VH and VK cDNA were cloned into M13-mpl9 vector. Clones were sequenced by the dideoxy method using T7 DNA Pol (Pharmacia). We reamplified the cDNA by PCR using VH1BACK and VH1FOR primers for VH and VK3BACK and VK3FOR primers for VK. The amplified cDNAs were digested with PstI and BstEII for the VH

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gene or PvuII and BgIII for the VK gene. The fragments were cloned into M13-VHPCR1 (digested with PstI and BstEII) or into M13-VKPCR1 (digested with PvuII and BclI). The M13VHPCR-R3 and M13VKPCR-R3 containing V gene inserts were identified directly by sequencing.

Construction of chimeric genes

[0034] The VH gene together with the Ig heavy chain promoter, appropriated splicing sites and signal peptide sequences were excised from M13 vectors by digestion with HindIII and BamHI and cloned into an expression vector (pSVgpt). A human IgG1 constant region (Takahashi N, Veda S, Obatu M, Nikaido T, Nakai S and Honjo T; (1982) Cell 29: 718-749), was then added as a BamHI fragment. The resultant construction was R3VH-pSVgpt. The construction of the R3VK-pSVhyg was essentially the same except that the gpt gene was replaced by the hygromycin resistance gene and a human Kappa chain constant region was added (Hieter PA, Max EE, Seidman JG, Maizel JV Jr and Leder P; (1980) Cell 22: 197-207).

Chimaeric antibody expresion

[0035] NSO cells were electroporated with 4 ug of R3VH-pSVgpt gamma 1 region and 8 ug R3VK-pSVhyg kappa constant region were linearized by digestion with PvuI. The DNAs were mixed together; ethanol precipitated and dissolved in 25 ul water. Approximately 10^7 NSO cells were grown to semiconfluency, harvested by centrifugation and resuspended in 0.5 ml DMEN together with the digested DNA in an electroporation cuvette. After 5 minutes on ice, the cells were given a pulse of 170 volts and 960 uF (Gene-Pulser, Bio-Rad) and left in ice for a further 30 minutes. The cells were then put into 20 ml DMEN plus 10% fetal calf serum and allowed to recover for 48 hours. At this time the cells were distributed into a 96-well plate and selective medium applied (DMEN, 10% fetal calf serum, 0.8 ug/ml mycophenolic acid, 250 ug/ml xanthine). Transfected clones were visible with the naked eye 14 days later.

[0036] The presence of human antibody in the medium of wells containing transfected clones was measured by ELISA. Microtiter plate wells were coated with goat anti-human IgG (gamma chain specific) antibodies (Sera Lab). After washing with PBST (phosphate buffered saline containing 0.02% Tween 20, pH 7.5), 20 ul of culture medium from the wells containing transfectants was added to each microtiter well for 1 hour at 37°C. The wells were then emptied, washed with PBST and peroxidase-conjugated goat anti-human Kappa, light chain specific (Sera-Lab), were added and incubated at 37°C for one hour. The wells were then emptied, washed with PBST and substrated buffer containing o-phenylediamine added. Reactions were stopped after a few minutes by the addition of sulphuric acid and absorbance at 492 nm was measured.

Transplantation of CDRs into Human Frameworks

[0037] The construction of the first version of humanized of R3-huVH(a) and R3-huVK was carried out using a CDR grafting approach similar to that described by Kunkel et al (Kunkel TA; (1985) Proc Natl Aca Sci USA 82, 488; Kunkel TA; (1987) Methods in Enzymology 155, 166). Briefly: To 0.5 ug of VH or VK single stranded Uracil DNA in M13 VH or VK PCR vectors (coding for the human Eu VH region sequence and human REI VK region sequence) were added ten pmoles of VH or VK phosphorylated oligonucleotides encoding the mouse CDRs sequences. Primers were annealed on the template by heating to 70°C and slowly cooled to 37°C. After site-directed mutagenesis, the DNA was transformed into competent E.coli TG1 cells. Single strand DNA was prepared from individual plaques and sequenced. If only single or double mutants were obtained, then these were subjected to further rounds of mutagenesis using the appropriated oligonucleotides until the triple CDR mutants were obtained.

[0038] Further versions of reshaped human R3VH were constructed using PCR mutagenesis (Kammann M, Laufs J, Schell J and Gronenborn B; (1989) PNAS USA 86, 4220-4224), using the oligonucleotides described in example 5.

Cloning and Expression of humanized R3 antibody into NSO cells

[0039] After CDR-grafting, the HindIII-BamHI fragment carrying the R3 humanized VH and R3 humanized VK genes were recloned in expression vectors, yielding the plasmids R3HuVH(1-7)-pSVgpt gammal and HuR3VK-pSVhyg kappa constant region. Vectors were linearized with PvuI, and humanized expression was done like chimaeric expression into NSO cells.

Molecular Modelling of mAb R3 VK and VH

[0040] A model of the variable regions of mouse mAb R3 was built using the molecular modeling program QUANTA/ CHARm 4.0 (Molecular Simulations Inc, 1994), running on a 150 MHz Silicon Graphics Indigo Extreme workstation.

The VK and VH frameworks were built separately from Fab 26-10 (Jeffrey PD, Strong RK, Sieker LC, Chang CY, Campbell RL, Petsko GA, Haber E, Margolies MN and Sheriff S; (1993) PNAS USA 90, 10310) and Fab 36-71 (Strong RK, Campbell RL, Rose DR, Petsko GA, Sharon J and Margolies MN; (1993) Biochemistry 30, 3739), respectively. Fab 26-10 and mAb R3 have 92% homology in the VK frameworks and 88% homology in the whole VK region. The VH frameworks of Fab 36-71 and R3 mAb have 85% homology.

[0041] Coordinates were taken from the Brookhaven Protein Data Bank (entries 1IGI and 6FAB). The frameworks of Fab 36-71 were fitted to the frameworks of Fab 26-10, matching only those residues that have been found to be often involved in the interface between the light and heavy variable regions (Chotia C, Novotny J, Bruccolery R and Karplus M; (1985) J Mol Biol 186, 651). The VH domain of Fab 26-10 and the VK domain of Fab 36-71 were then deleted leaving the needed hybrid. Side-chain replacements were performed following the maximum overlap procedure (Snow ME and Amzel LM; (1986) Proteins 1, 267), and comparing, where possible, with other crystal structures.

[0042] The hypervariable regions of the R3-Variable Light (VL) domain (L1, L2 and L3) were built retaining the same main-chain conformations as in Fab 26-10, since the corresponding CDRs in both antibodies are highly homologous and belong to the same canonical structural groups (Chotia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, Air G, Sherii S, Padlan EA, Davies D, Tulip WR, Colman PM, Spinelli S, Alzari PM and Poljak RJ; (1989) Nature 342, 877). In the VH domain of mAb R3, CDR H1 belongs to canonical structural group 1, as in Fab 36-71, so the main-chain torsion angles of the parent molecule were kept. CDR H2 corresponds to canonical structural group 2 and the main-chain conformation for this loop was taken from the Fv fragment 4D5 (entry 1FVC), which was selected among other highly resolved structures because of the good matching of its H2 loop base with the framework of Fab 36-71. For all the above mentioned loops comparisons with other CDRs from the Data Bank were made to orient the side chains.

[0043] To model CDR H3, which in mAb R3 was 14 amino acids long, a high temperature molecular dynamics was used for conformational sampling (Bruccoleri RE and Karplus M; (1990) Biopolymers 29, 1847). First, the whole structure without CDR H3 was subjected to an energy minimization keeping residues H-94 and H-103 fixed and using harmonic constraints of 10 Kcal/(mole atom A^2) for main chain atoms. Then a loop was constructed with an arbitrary conformation starting from the two previously fixed amino acids. Those residues close to the framework were placed taking into consideration other crystal structures and the top part of the loop was built with an extended conformation avoiding strong steric interactions with the rest of the molecule. For the next modeling steps only CDR H3 and the neighboring side chains within a distance of $5A^0$ were permitted to move. An energy minimization was first carried out and then a molecular dynamics at 800 K was run for 150 picoseconds. The time step for the run was set to 0.001 picosecond and coordinates were saved every 100 steps. The 120 lowest energy conformations from the dynamics run were extracted and subjected to an energy minimization in which all atoms in the structure were allowed to move. Several low-energy conformations were obtained and the one with the lowest energy was used in the subsequent analyses. Differences between murine and humanized variants of R3 antibody were individually modeled to investigate their possible influence on CDR conformation.

EGF Receptor Radioligand Competition assays

[0044] The determination of the apparent inhibition constants (Ki) of the ^{125}I-EGF binding to its receptor by the anti EGF-R mAb was performed by an homogeneous Radio Receptor Analysis (RRA) with human placenta microsomal fraction (Macias A, Pérez R, Lage A; (1985) Interferon y Biotecnologia 2: 115-127). The affinity constant of the antigen antibody reaction was estimated also by a competitive RRA but using mAb ^{125}I-R3 as the radiolabelled probe.

Materials

[0045] Recombinant Human Epidermal Growth Factor (hEGF) was obtained from the Center of Genetic Engineering and Biotechnology, Havana, Cuba. ^{125}I-hEGF was radioiodinated by the chloramine-T method (specific activity 150-200 uCi/ug). Murine hybridoma cell line R3 was obtained by us (EPA No 93202428.4). Rat myeloma NSO is a non-Ig secreting cell line and was grown in Dulbecco's modified Eagles medium (DMEN) containing 10% fetal calf serum. Vectors M13VHPCR1, M13VKPCR1, pSVgpt and pSVhyg have been described in detail (Orlandi R, Gussow DH, Jones PT and Winter G; (1989) Proc Natl Acad Sci USA 86: 3833-3837) and were obtained from Greg Winter, MRC Laboratory of Molecular Biology, Cambridge, UK. Oligonucleotides were synthesized using an Applied Biosystems 381 DNA synthesizer.

Pharmaceutical compositions

[0046] With respect to the formulation of suitable compositions for administration to patients in need of treatment, the monoclonal antibodies according to the invention may be admixed or combined with pharmaceutically acceptable carriers known per se, dependent upon the chosen route of administration. There are no particular limitations to the

modes of application of the invention, and the choice of suitable administration routes and suitable compositions belong to the routine skills of persons skilled in the art.

[0047] Although other forms of administration are possible as well, a preferred administration form would be a solution for injection, in particular for intravenous or intraarterial injection. Usually, a suitable pharmaceutical composition for injection may comprise a buffer (e.g. acetate, phosphate or citrate buffer), a surfactant (e.g. polysorbate), optionally a stabilizer agent (e.g. human albumine), etc. Persons skilled in the art know, or can easily determine, other possibilities.

[0048] Similarly, persons skilled in the art have the ability to determine the best concentrations and proportions of the various components, administration dose and frequency, etc. For example, a suitable solution for injection will usually contain from about 1 to about 20, preferably 5-10 mg antibody per ml. The dose to be administered could for example be from about 0.1 to about 20, preferably 1-5 mg/kg body weight, and administration could be once per day, or three times per week, or whatever the physician thinks best.

EXAMPLE 1

Molecular Cloning sequencing

[0049] VH and VK were amplified using PCR. The specific oligonucleotides used as primers were:

For the heavy chain variable region:

CG2AFOR (5' GGAAGCTTAGACCGATGGGGCCTGTTGTTTTG 3');

VH1BACK (5' AGGT(G/C)(A/C)A(A/G)CTGCAG(G/C)AGTC(A/T)GG 3').

For the light chain variable region:

CK2FOR (5' GGAAGCTTGAAGATGGATACAGTTGGTGCAGC 3');

VK10BACK (5' TTGAATTCCAGTGATGTTTTGATGACCCA 3').

[0050] The purified VH and VK cDNA were cloned into M13 vector. Twelve independent clones were sequenced by the dideoxy method using T7 DNA Pol (Pharmacia). In figure 1, you can see the variable region sequences of the murine R3 mAb. The VH sequence is most closely related to kabat subgroup VH IIB and the VK to kabat subgroup II.

EXAMPLE 2

Construction of chimeric genes

[0051] We reamplified the cDNA by PCR using the following oligonucleotides as primers:

For VH:

VH1BACK (5' AGGT(G/C)(A/C)A(A/G)CTGCAG(G/C)AGTC(A/T)GG 3');

VH1FOR (5' TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG 3').

For Vk:

VK3BACK (5' GACATTCAGCTGACCCA 3');

VK3FOR (5' GTTAGATCTCCAGTTTGGTGCT 3').

[0052] The amplified cDNAs were digested with PstI and BstEII for the VH gene or PvuII and BglII for the VK gene. The fragments were cloned into M13-VHPCR1 (digested with PstI and BstEII) or into M13-VKPCR1 (digested with PvuII and BclI). Details of vectors (Orlandi R et al; Proc Natl Acad Sci USA 86: 3833-3837, 1989). The M13VHPCR-R3

and M13VKPCR-R3 containing V gene inserts were identified directly by sequencing.

[0053] The VH gene together with the Ig heavy chain promoter, appropriated splicing sites and signal peptide sequences were excised from M13 vectors by digestion with HindIII and BamHI and cloned into an expression vector (pSVgpt). A human IgGl constant region (Takahashi N et al; Cell 29: 718-749, 1982) was then added as a BamHI fragment. The resultant construction was R3VH-pSVgpt. The construction of the R3VK-pSVhyg was essentially the same, except that the gpt gene was replaced by the hygromycin resistance gene and a human Kappa chain constant region was added (Hieter PA et al; Cell 22: 197-207, 1980).

EXAMPLE 3

Transplantation of murine CDRs of R3 into human FRs

[0054] We compared murine light and heavy chains variable regions of R3 with variable regions sequences of human immunoglobulins.

[0055] The murine R3 heavy chain variable region has 63.4% of homology with human subgroup VHI and the R3 light chain variable region has 63.7% of homology with human subgroup VKI. The human FRs choosen were derived of the human immunoglobulin REI for the light chain and the human immunoglobulin EU for the heavy chain.

[0056] The construction of the first version of the humanized R3 heavy chain and humanized R3 light chain was carried out using CDR grafting approach as described in Detailed description of the invention.

[0057] Oligonucleotides were designed which consisted of DNA sequences coding for each end by 12 bases of DNA complementary to the DNA sequences coding for the adjacent FRs of human Eu for Vh and human REI for VK.

[0058] The oligonucleotides designed were:

For the CDR1 of the light chain:

5' TCTAGATCAGTCTTGTAACATGTATCATTACCTTTGTGGATAAATCTG 3'.

[0059] We didn't use oligonucleotides for the CDR2 because REI human CDR2 is identical to R3 CDR2.

For the CDR3 of the ligh chain:

5' GATGACGAAAGTTATCAAGTGTACAAGGGACCTG 3'.

For the CDR1 of the heavy chain:

5' ATGTGGAAGTGGTTAATAATATAGATAACCCACTCTGTC 3'.

For the CDR2 of the heavy chain:

5' ACTACCTACCCTCCCTATTTGGGGTGGAGACCTCCCTCATTGAAATTACTTTTCAGTTC
TGTTCTCACTGTTAA 3'.

For the CDR 3 of the heavy chain:

5' AAAACACGTTCTGTCCCGAACACCAAGCTGTCACTGCCTGCCCCGAAACTGAAG 3'.

EXAMPLE 4

Expression of chimeric and humanized in NSO cells

[0060] NSO cells were electroporated with 4 ug of murine or humanized R3VH-CMMAR gamma 1 region and 8 ug of murine or humanized R3VK-CMMARhyg kappa constant region were linearized by digestion with PvuI. The DNAs were mixed together, ethanol precipitated and dissolved in 25 ul water. Approximately 10^7 NSO cells were grown to

semiconfluency, harvested by centrifugation and resuspended in 0.5 ml DMEN together with the digested DNA in an electroporation cuvette. After 5 minutes on ice, the cells were given a pulse of 170 volts and 960 uF (Gene-Pulser, Bio-Rad) and left in ice for a further 30 minutes. The cells were put into 20 ml DMEN plus 10% fetal calf serum and allowed to recover for 48 hours. At this time the cells were distributed into 96-well plate and selective medium applied (DMEN, 10% fetal calf serum, 0.8 ug/ml mycophenolic acid, 250 ug/ml xanthine). Transfected clones were visible with the naked eye 14 days later.

[0061] The presence of human antibody in the medium of wells containing transfected clones was measured by ELISA. Microtiter plate wells were coated with goat anti-human IgG (gamma chain specific) antibodies (Sera Lab). After washing with PBST (phosphate buffered saline containing 0.02% Tween 20, pH 7.5), 20 ul of culture medium from the wells containing transfectants was added to each microtiter well for 1 hour at 37°C. The wells were then emptied, washed with PBST and peroxidase-conjugated goat anti-human Kappa, light chain specific (Sera-Lab), were added and incubated at 37°C for one hour. The wells were then emptied, washed with PBST and substrated buffer containing o-phenylenediamine added. Reactions were stopped after a few minutes by the addition of sulphuric acid and absorbance at 492 nm was measured.

[0062] In figure 3 you can see that the chimeric and murine R3 antibody bind to the EGF-R with the same affinity (tested in RRA), and the first version of humanized R3 antibody didn't bind to the EGF-R.

EXAMPLE 5

Construction of different versions of humanized R3 antibodies, with some murine residues in the FRs regions

[0063] The introduction of some murine residues in the human FRs was necessary because the first version of humanized that we constructed didn't bind to the antigen.

[0064] We constructed hybrids between murine R3VH/humanized R3VK and humanized R3VH/murine R3VK. Figure 4 shows that the hybrid murine R3VH/humanized R3VK binds to the EGF-R with the same affinity of original murine R3.

[0065] Humanized R3huVk retained all the Vernier's residues of murine R3VK. This fact may explain why the humanizing procedure did not affect the binding capability of the kappa chain.

[0066] Nevertheless humanized R3VH/murine R3VK didn't work. This result suggested us that we had to include murine residues into human FRs of the heavy chain, for the recovery of the binding to the antigen of this antibody.

[0067] Then, other versions of the reshaped humanized heavy chains were constructed by PCR mutagenesis. In these new versions we tried to remain intact the Vernier's zone, recommending changes in the position 66, 67, 75, 76 and 93 in the FRs regions.

[0068] The oligonucleotides designed to get the reshaped R3VH-K66A67 were:

Top strand:

5' GAAAAGTTCAAGACAAAAGCGACAATTACGGTAGAC 3'.

Lower strand:

5' GTCTACCGTAATTGTCGCTTTTGTCTTGAACTTGAACTTTTC 3'.

[0069] The oligonucleotides designed to get the reshaped R3VH-S75T76 were:

Top strand:

5' GTAGACGAGAGCAGCACCACGGCGTACATG 3'.

Lower strand:

5' CATGTACGCCGTGGTGCTGCTCTCGTCTAC 3'.

[0070] The oligonucleotides designed to get the reshaped R3VH-T93 were:

Top strand:

·5' TTCTATTTTTGTACAAGACAGGGCTTG 3'.

Lower strand:

5' CAAGCCCTGTCTTGTACAAAAATAGAA 3'.

EXAMPLE 6

Molecular Modelling of mAb R3 VK and VH

[0071] A model of the variable regions of mouse mAb R3 was built using the molecular modeling program QUANTA/ CHARm 4.0 (Molecular Simulations Inc, 1994), running on a 150 MHz Silicon Graphics Indigo Extreme workstation.
[0072] Differences between murine and humanized variants of R3 antibody were individually modeled to investigate their possible influence on CDR conformation.
[0073] Heavy chain variable region residues at positions 66, 67, 75, 76 and 93, which had been changed in humanized R3huVH (figure 6), are close to the hypervariable loops and therefore may influence the CDR conformations. Both our experimental results and the modeling studies suggest that only Thr76 and Thr93 are critical for binding affinity.
[0074] A molecular model of the murine R3 heavy chain variable regions was constructed to analyze the possible effects of these mutations. Residue 93 was located just below CDR H3, close to Phe100f (fig 6), and replacing Thr by a smaller amino acid like Ala may provoke some rearrangements of the neighboring side chains and modify the overall conformation of the H3 loop.
[0075] THR 76 as close to CDR H2 (fig 2) and the introduction of the larger Asn residue as this position could lead to hydrogen-bonding interactions with the backbone of CDR H1. Moreover, residue 76 is accessible from the top of the variable region and could be directly involved in the binding to EGF-R. The substitution Ser75--Thr alone did not seem to have any influence, but taken together with mutation at position 76, it could be important. The changes Lys66-- Arg and Ala67--Val were not seen to affect the structure, but since they had been found to have some influence in the functional binding of reshaped mAb 425 (Kettleborough C, Saldanha J, Heath VJ, Morrison CJ and Bending MM; (1991) Protein Engineering 4, 773-783), we decided to make substitutions at these positions as well.
[0076] Humanized R3huVK retained all the Vernier's residues of murine R3VK. This fact may explain why the humanizing procedure did not affect the binding capability of the kappa chain.
[0077] Reshaped R3 hu VH antibodies containing either S75/T76 or T93 recovered partially the binding capacity, whereas those constructs containing both of them retained full binding activity (fig 7).

EXAMPLE 7

EGF Receptor Radioligand Competition assays

[0078] The determination of the affinity constant of the ^{125}I-EGF binding to its receptor by murine R3, chimeric and humanized antibodies was performed by an homogeneous Radio Receptor Analysis (RRA) with human placenta microsomal fraction (Macias A et al; Interferon y Biotecnologia 2: 115-127, 1985).
[0079] These chimeric and humanized different versions antibodies were assayed using this technique for its ability to bind to EGF-R (figure 7). As you can see the different versions of reshaped human VH regions result in a wide variety of levels of antigen binding (figure 7). Versions 6 and 7 have the same affinity of original murine antibody, these versions with highest levels of binding were followed of versions 3, 4 and 5 and then followed by version 2.
[0080] Based on these results, it is possible to comment on the relative contributions of individual residues in the FRs make to antigen binding. The 75 and 76 changes are together with 93 very important to the binding, while the introduction of 66 and 67 changes fail to produce significant antigen binding.

EXAMPLE 8

IMMUNIZATION OF CERCOPITHECUS AETHIOPS MONKEYS WITH THE MURINE, CHIMERIC AND VH MUTANT ANTIBODIES

[0081] Three treatment groups with two cercopithecus aethiops monkeys in each group were immunized as follows:

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1.- Murine R3 monoclonal antibody (2 mg) with 5 mg of aluminum hydroxide as adjuvant; 2.- Chimeric R3 antibody (2 mg) with 5 mg of aluminum hydroxide as adjuvant; and 3.- Humanized (version 6) R3 antibody (2 mg) with 5 mg of aluminum hydroxide as adjuvant. All the groups were immunized intradermically on weeks 1, 3, 5, and 7. Starting on week one, blood was collected from all the groups weekly.

[0082] The serum obtained and the titer of antibodies against EGF-R was determined by an ELISA technique.

[0083] Costar plates (Inc, high binding) were coated with murine R3 monoclonal antibody at a concentration of 10 ug/ml in bicarbonate buffer (pH 9.6) and incubated overnight. After, the plates were washed with PBST, were blocked with the same buffer containing 1% BSA during one hour at room temperature.

[0084] Washing step was repeated and 50ul/well of the different serum dilutions were added. After incubating for 2 hours at 37°C, the plates were washed again and incubated 1 hour at 37°C with alkaline phosphated conjugated goat anti-human total or anti-human IgG Fc region specific antiserum (Sigma, Inc). After washing with PBST the wells were incubated with 50 ul of substrate buffer (1 mg/ml of p-nitrophenylphosphate diluted in diethanolamine buffer (pH 9.8) and absorbance at 405 nm was read with an ELISA reader (Organon Teknika, Inc).

[0085] A high IgG response to murine R3 antibody was obtained when this antibody was used as immunogen. A lower but still measurable IgG response (1/10000) to the murine R3 antibody was obtained when monkeys were immunized with the chimeric antibody, contrary to the results obtained with the humanized (version 6), (figure 8). With the humanized antibody no response was measurable after 3 immunizations.

DEPOSIT

[0086] The cell line hr-R3 expressing the antibody was deposited pursuant to the Budapest Treaty with European Collection of Cell Cultures (CAMR, Salisbury, Wiltshire SP4, OJG, UK), and obtained deposit number 951110101.

Claims

1. A monoclonal antibody which specifically binds epidermal growth factor receptor (EGF-R), **characterized in that** it is a chimeric or a humanized antibody which contains the following complementarity determining regions (CDRs) :

> CDR1 of the light chain: **RSSQNIVHSNGNTYLD,**
> CDR2 of the light chain: **KVSNRFS,**
> CDR3 of the light chain: **FQYSHVPWT,**
> CDR1 of the heavy chain: **NYYIY,**
> CDR2 of the heavy chain: **GINPTSGGSNFNEKFKT,**
> CDR3 of the heavy chain: **QGLWFDSDGRGFDF.**

2. A monoclonal antibody according to claim 1, wherein said antibody is a chimeric antibody having the following variable regions of the light and heavy chains:

> variable region of the light chain:

DVLMTQIPLSLPVSLGDQASISC**RSSQNIVHSNGNTYLD**WYLQKPGQSPNLLIY**KVSNRFS**SG
VPDRFRGSGSGTDFTLKISRVEAEDLGVYYC**FQYSHVPWT**FGGGTKLEIKRA;

> variable region of the heavy chain:

QVQLQQPGAELVKPGASVKLSCKASGYTF**TNYYIY**WVKQRPGQGLEWIG**GINPTSGGSNFNE**
KFKTKATLTVDESSTTAYMQLSSLTSEDSAVYYCTR**QGLWFDSDGRGFDF**WGQGTTLTVSS.

3. A monoclonal antibody according to claim 1, wherein said antibody is a humanized antibody having the following variable regions of the light and heavy chains:

> variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITC**RSSQNIVHSNGNTYLD**WYQQTPGKAPKLLIY**KVSNRFSG**
VPSRFSGSGSGTDFTFTISSLQPEDIATYYC**FQYSHVPWT**FGQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFT**NYYIY**WVRQAPGQGLEWIG**GINPTSGGSNFNE**

KFKTx^{1}x^{2}TITVDES**x**^{3}**x**^{4}TAYMELSSLRSEDTAFYFCx^{5}R**QGLWFDSDGRGFDF**WGQGSTVTVSS,

wherein x^1 is R or K; x^2 is V or A; x^3 is T or S; x^4 is N or T; and x^5 is A or T.

4. A monoclonal antibody according to claim 3, wherein said antibody is a humanized antibody having the following variable regions of the light and heavy chains:

variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITC**RSSQNIVHSNGNTYLD**WYQQTPGKAPKLLIY**KVSNRFSG**
VPSRFSGSGSGTDFTFTISSLQPEDIATYYC**FQYSHVPWT**FGQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFT**NYYIY**WVRQAPGQGLEWIG**GINPTSGGSNFNE**
KFKTRVTITVDESSTTAYMELSSLRSEDTAFYFCTR**QGLWFDSDGRGFDF**WGQGSTVTVSS.

5. A monoclonal antibody according to claim 3, wherein said antibody is a humanized antibody having the following variable regions of the light and heavy chains:

variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITC**RSSQNIVHSNGNTYLD**WYQQTPGKAPKLLIY**KVSNRFSG**
VPSRFSGSGSGTDFTFTISSLQPEDIATYYC**FQYSHVPWT**FGQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFT**NYYIY**WVRQAPGQGLEWIG**GINPTSGGSNFNE**
KFKTKATITVDESSTTAYMELSSLRSEDTAFYFCTR**QGLWFDSDGRGFDF**WGQGSTVTVSS.

6. A monoclonal antibody according to claim 3, wherein said antibody is a humanized antibody having the following variable regions of the light and heavy chains:

variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITC**RSSQNIVHSNGNTYLD**WYQQTPGKAPKLLIY**KVSNRFSG**
VPSRFSGSGSGTDFTFTISSLQPEDIATYYC**FQYSHVPWT**FGQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFT**NYYIY**WVRQAPGQGLEWI**GGINPTSGGSNFNE**
KFKTRVTITVDESSTTAYMELSSLRSEDTAFYFCAR**QGLWFDSDGRGFDF**WGQGSTVTVSS.

7. A monoclonal antibody according to claim 3, wherein said antibody is a humanized antibody having the following variable regions of the light and heavy chains:

variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITC**RSSQNIVHSNGNTYLD**WYQQTPGKAPKLLIY**KVSNRFSG**
VPSRFSGSGSGTDFTFTISSLQPEDIATYYC**FQYSHVPWT**FGQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFT**NYYIY**WVRQAPGQGLEWI**GGINPTSGGSNFNE**
KFKTRVTITVDESTNTAYMELSSLRSEDTAFYFCTR**QGLWFDSDGRGFDF**WGQGSTVTVSS.

8. A monoclonal antibody according to claim 3, wherein said antibody is a humanized antibody having the following variable regions of the light and heavy chains:

variable region of the light chain:

DIQMTQSPSSLSASVGDRVTITC**RSSQNIVHSNGNTYLD**WYQQTPGKAPKLLIY**KVSNRFSG**
VPSRFSGSGSGTDFTFTISSLQPEDIATYYC**FQYSHVPWT**FGQGTKLQITRE;

variable region of the heavy chain:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFT**NYYIY**WVRQAPGQGLEWI**GGINPTSGGSNFNE**
KFKTKATITVDESTNTAYMELSSLRSEDTAFYFCTR**QGLWFDSDGRGFDF**WGQGSTVTVSS.

9. A monoclonal antibody according to any one of claims 1-8, wherein the constant regions of the heavy chain of said antibody comprise the amino acid sequence of a gamma-1 chain of a human immunoglobulin and the constant regions of the light chain of said antibody comprise the amino acid sequence of a kappa chain of a human immunoglobulin.

10. A monoclonal antibody according to claim 1, which is the antibody produced by the cell line hr-R3 deposited as ECACC 951110101.

11. A cell line producing a monoclonal antibody, wherein said cell line is hr-R3 deposited as ECACC 951110101.

12. A pharmaceutical composition comprising a monoclonal antibody according to any one of the claims 1-10 and a pharmaceutically acceptable carrier.

13. A pharmaceutical composition according to claim 12 for use against tumors.

14. A diagnostic reagent for localization and assessment of growth of a tumor, comprising a monoclonal antibody according to any one of the claims 1-10.

Patentansprüche

1. Monoklonaler Antikörper, der den Rezeptor des epidermalen Wachstumsfaktors (EGF-R) spezifisch bindet, **da-**

durch gekennzeichnet, dass es sich dabei um einen chimären oder humanisierten Antikörper handelt, der die folgenden komplementätsbestimmenden Bereiche (CDR) enthält:

den CDR1 der leichten Kette: **RSSQNIVHSNGNTYLD,**

den CDR2 der leichten Kette: **KVSNRFS,**

den CDR3 der leichten Kette: **FQYSHVPWT,**

den CDR1 der schweren Kette: **NYYIY,**

den CDR2 der schweren Kette: **GINPTSGGSNFNEKFKT,**

den CDR3 der schweren Kette: **QGLWFDSDGRGFDF.**

2. Monoklonaler Antikörper nach Anspruch 1, wobei der Antikörper ein chimärer Antikörper mit den folgenden variablen Bereichen der leichten und der schweren Kette ist:

variabler Bereich der leichten Kette:

DVLMTQIPLSLPVSLGDQASISCRSSQNIVHSNGNTYLDWYLQKPGQSPNLLIYKVSNRFSG VPDRFRGSGSGTDFTLKISRVEAEDLGVYYCFQYSHVPWTFGGGTKLEIKRA;

variabler Bereich der schweren Kette:

QVQLQQPGAELVKPGASVKLSCKASGYTFTNYYIYWVKQRPGQGLEWIGGINPTSGGSNFNE KFKTKATLTVDESSTTAYMQLSSLTSEDSAVYYCTRQGLWFDSDGRGFDFWGQGTTLTVSS.

3. Monoklonaler Antikörper nach Anspruch 1, wobei der Antikörper ein humanisierter Antikörper mit den folgenden variablen Bereichen der leichten und der schweren Kette ist:

variabler Bereich der leichten Kette:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLLIYKVSNRFSG VPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKLQITRE;

variabler Bereich der schweren Kette:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGINPTSGGSNFNE KFKTx^{1}x^{2}TITVDESx^{3}x^{4}TAYMELSSLRSEDTAFYFCx^{5}RQGLWFDSDGRGFDFWGQGSTVTVSS,

wobei x^1 R oder K ist; x^2 V oder A ist; x^3 T oder S ist; x^4 N oder T ist und x^5 A oder T ist.

4. Monoklonaler Antikörper nach Anspruch 3, wobei der Antikörper ein humanisierter Antikörper mit den folgenden variablen Bereichen der leichten und der schweren Kette ist:

variabler Bereich der leichten Kette:

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DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLLIYKVSNRFSG
VPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKLQITRE;

variabler Bereich der schweren Kette:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGINPTSGGSNFNE
KFKTRVTITVDESSTTAYMELSSLRSEDTAFYFCTRQGLWFDSDGRGFDFWGQGSTVTVSS.

5. Monoklonaler Antikörper nach Anspruch 3, wobei der Antikörper ein humanisierter Antikörper mit den folgenden variablen Bereichen der leichten und der schweren Kette ist:

variabler Bereich der leichten Kette:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLLIYKVSNRFSG
VPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKLQITRE;

variabler Bereich der schweren Kette:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGINPTSGGSNFNE
KFKTKATITVDESSTTAYMELSSLRSEDTAFYFCTRQGLWFDSDGRGFDFWGQGSTVTVSS.

6. Monoklonaler Antikörper nach Anspruch 3, wobei der Antikörper ein humanisierter Antikörper mit den folgenden variablen Bereichen der leichten und der schweren Kette ist:

variabler Bereich der leichten Kette:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLLIYKVSNRFSG
VPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKLQITRE;

variabler Bereich der schweren Kette:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGINPTSGGSNFNE
KFKTRVTITVDESSTTAYMELSSLRSEDTAFYFCARQGLWFDSDGRGFDFWGQGSTVTVSS.

7. Monoklonaler Antikörper nach Anspruch 3, wobei der Antikörper ein humanisierter Antikörper mit den folgenden variablen Bereichen der leichten und der schweren Kette ist:

variabler Bereich der leichten Kette:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLLIYKVSNRFSG
VPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKLQITRE;

variabler Bereich der schweren Kette:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGINPTSGGSNFNE
KFKTRVTITVDESTNTAYMELSSLRSEDTAFYFCTRQGLWFDSDGRGFDFWGQGSTVTVSS.

8. Monoklonaler Antikörper nach Anspruch 3, wobei der Antikörper ein humanisierter Antikörper mit den folgenden variablen Bereichen der leichten und der schweren Kette ist:

variabler Bereich der leichten Kette:

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLLIYKVSNRFSG
VPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKLQITRE;

variabler Bereich der schweren Kette:

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGINPTSGGSNFNE
KFKTKATITVDESTNTAYMELSSLRSEDTAFYFCTRQGLWFDSDGRGFDFWGQGSTVTVSS.

9. Monoklonaler Antikörper nach einem der Ansprüche 1 - 8, wobei die konstanten Bereiche der schweren Kette des Antikörpers die Aminosäuresequenz einer γ-1-Kette eines humanen Immunoglobulins umfassen und die konstanten Bereiche der leichten Kette des Antikörpers die Aminosäuresequenz einer κ-Kette eines humanen Immunoglobulins umfassen.

10. Monoklonaler Antikörper nach Anspruch 1, bei dem es sich um den Antikörper handelt, der von der Zelllinie hr-R3, hinterlegt als ECACC 951110101, produziert wird.

11. Zelllinie, die einen monoklonalen Antikörper produziert, wobei es sich bei der Zelllinie um hr-R3, hinterlegt als ECACC 951110101, handelt.

12. Pharmazeutische Zusammensetzung, umfassend einen monoklonalen Antikörper nach einem der Ansprüche 1 - 10 und einen pharmazeutisch annehmbaren Träger.

13. Pharmazeutische Zusammensetzung nach Anspruch 12 zur Verwendung gegen Tumore.

14. Diagnosereagens zur Lokalisierung und Bewertung des Wachstums eines Tumors, umfassend einen monoklonalen Antikörper nach einem der Ansprüche 1 - 10.

Revendications

1. Anticorps monoclonal qui fixe spécifiquement le récepteur de facteur de croissance épidermique (EGF-R), **caractérisé en ce qu'**il s'agit d'un anticorps chimérique ou humanisé qui contient les régions déterminantes complémentaires (CDR) suivantes :

CDR1 de la chaîne légère : RSSQNIVHSNGNTYLD,
CDR2 de la chaîne légère : KVSNRFS,
CDR3 de la chaîne légère : FQYSHVPWT,
CDR1 de la chaîne lourde : NYYIY,
CDR2 de la chaîne lourde : GINPTSGGSNFNEKFKT,
CDR3 de la chaîne lourde : QGLWFDSDGRGFDF

2. Anticorps monoclonal selon la revendication 1, dans lequel ledit anticorps est un anticorps chimérique ayant les régions variables suivantes dans les chaînes lourde et légère :

région variable de la chaîne légère :

DVLMTQIPLSLPVSLGDQASISCRSSQNIVHSNGNTYLDWYLQKPGQSPNLL
IYKVSNRFSGVPDRFRGSGSGTDFTLKISRVEAEDLGVYYCFQYSHVPWTFGGGTKL
EIKRA

région variable de la chaîne lourde :

QVQLQQPGAELVKPGASVKLSCKASGYTFTNYYIYWVKQRPGQGLEWIGGIN
PTSGGSNFNEKFKTKATLTVDESSTTAYMQLSSLTSEDSAVVYCTRQGLWFDSDGRG
FDFWGQGTTLTVSS

3. Anticorps monoclonal selon la revendication 1, dans lequel ledit anticorps est un anticorps humanisé ayant les régions variables suivantes des chaînes lourde et légère :

région variable de la chaîne lourde :

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLL

IYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKL
QITRE

région variable de la chaîne lourde :

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGIN
PTSGGSNFNEKFKTx^1x^2TITVDESx^3x^4TAYMELSSLRSEDTAFYFCx^5RQGLWFDS
DGRGFDFWGQGSTVTVSS

où x^1 est R ou K ; x^2 est V ou A ; x^3 est T ou S ; x^4 est N ou T ; et x^5 est A ou T.

4. Anticorps monoclonal selon la revendication 3, dans lequel ledit anticorps est un anticorps humanisé ayant les régions variables suivantes des chaînes lourde et légère :

région variable de la chaîne lourde :

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLL
IYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKL
QITRE

région variable de la chaîne lourde :

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGIN
PTSGGSNFNEKFKTRVTITVDESSTTAYMELSSLRSEDTAFYFCTRQGLWFDSDGRG
FDFWGQGSTVTVSS

5. Anticorps monoclonal selon la revendication 3, dans lequel ledit anticorps est un anticorps humanisé ayant les régions variables suivantes des chaînes lourde et légère :

    région variable de la chaîne lourde :

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLL
IYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKL
QITRE

    région variable de la chaîne lourde :

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGIN
PTSGGSNFNEKFKTKATITVDESSTTAYMELSSLRSEDTAFYFCTRQGLWFDSDGRG
FDFWGQGSTVTVSS

6. Anticorps monoclonal selon la revendication 3, dans lequel ledit anticorps est un anticorps humanisé ayant les régions variables suivantes des chaînes lourde. et légère :

    région variable de la chaîne lourde :

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLL
IYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKL
QITRE

    région variable de la chaîne lourde :

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGIN
PTSGGSNFNEKFKTRVTITVDESSTTAYMELSSLRSEDTAFYFCARQGLWFDSDGRG
FDFWGQGSTVTVSS

7. Anticorps monoclonal selon la revendication 3, dans lequel ledit anticorps est un anticorps humanisé ayant les régions variables suivantes des chaînes lourde et légère :

    région variable de la chaîne lourde :

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLL

IYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKL

QITRE

région variable de la chaîne lourde :

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGIN

PTSGGSNFNEKFKTRVTITVDESTNTAYMELSSLRSEDTAFYFCTRQGLWFDSDGRG

FDFWGQGSTVTVSS

8. Anticorps monoclonal selon la revendication 3, dans lequel ledit anticorps est un anticorps humanisé ayant les régions variables suivantes des chaînes lourde et légère :

région variable de la chaîne lourde :

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLL

IYKVSNRFSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKL

QITRE

région variable de la chaîne lourde :

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGIN

PTSGGSNFNEKFKTKATITVDESTNTAYMELSSLRSEDTAFYFCTRQGLWFDSDGRG

FDFWGQGSTVTVSS

9. Anticorps monoclonal selon l'une quelconque des revendications 1 à 8, dans lequel les régions constantes de la chaîne lourde dudit anticorps comprennent la séquence d'acides aminés d'une chaîne gamma-1 d'une immuno-globuline humaine et les régions constantes de la chaîne légère dudit anticorps comprennent la séquence d'acides aminés d'une chaîne kappa d'une immunoglobuline humaine.

10. Anticorps monoclonal selon la revendication 1, qui est l'anticorps produit par la lignée cellulaire hr-R2 déposée sous la référence ECACC 951110101.

11. Lignée cellulaire produisant un anticorps monoclonal, dans laquelle ladite lignée cellulaire est la hr-23 déposée sous la référence ECACC 951110101.

12. Composition pharmaceutique comprenant un anticorps monoclonal selon l'une quelconque des revendications 1 à 10 et un véhicule acceptable en pharmacie.

13. Composition pharmaceutique selon la revendication 12, à utiliser contre des tumeurs.

14. Réactif diagnostique pour la localisation et la détermination de la croissance d'une tumeur, comprenant un anti-corps monoclonal selon l'une quelconque des revendications 1 à 10.

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FIGURE 1: Deduced aminoacid sequence of (a) VK and (b) VH of
murine R3 antibody. CDRs are underlined.

a)

```
D V L M T Q I P L S L P V S L G D Q A S I S C R S S Q N I
V H S N G N T Y L D W Y L Q K P G Q S P N L L I Y K V S N
R F S G V P D R F R G S G S G T D F T L K I S R V E A E D
L G V Y Y C F Q Y S H V P W T F G G G T K L E I K R A
```

b)

```
Q V Q L Q Q P G A E L V K P G A S V K L S C K A S G Y T F
T N Y Y I Y W V K Q R P G Q G L E W I G G I N P T S G G S
N F N E K F K T K A T L T V D E S S T A Y M Q L S S L T
S E D S A V Y Y C T R Q G L W F D S D G R G F D F W G Q G
T T L T V S S
```

FIGURE 2: Aminoacid sequences of murine and humanized VK(a) and
VH(b) containing mAb R3 CDRs.

a)

```
murine VkR3    D V L M T Q I P L S L P V S L G D Q A S I S C
human. VkR3    D I Q M T Q S P S S L S A S V G D R V T I T C

murine VkR3    R S S Q N I V H S N G N T Y L D W Y L Q K P G
human. VkR3    R S S Q N I V H S N G N T Y L D W Y Q Q T P G

murine VkR3    Q S P N L L I Y K V S N R F S G V P D R F R G
human. VkR3    K A P K L L I Y K V S N R F S G V P S R F S G

murine VkR3    S G S G T D F T L K I S R V E A E D L G V Y Y
human. VkR3    S G S G T D F T F T I S S L Q P E D I A T Y Y

murine VkR3    C F Q Y S H V P W T F G G G T K L E I K R A
human. VkR3    C F Q Y S H V P W T F G Q G T K L Q I T R E
```

b)

```
murine VHR3    Q V Q L Q Q P G A E L V K P G A S V K L S C K A
human. VHaR3   Q V Q L Q Q S G A E V K K P G S S V K V S C K A

murine VHR3    S G Y T F T N Y Y I Y W V K Q R P G Q G L E W I
human. VHaR3   S G Y T F T N Y Y I Y W V R Q A P G Q G L E W I

murine VHR3    G G I N P T S G G S N F N E K F K T K A T L T V
human. VHaR3   G G I N P T S G G S N F N E K F K T R V T I T V

murine VHR3    D E S S T T A Y M Q L S S L T S E D S A V Y Y C
human. VHaR3   D E S T N T A Y M E L S S L R S E D T A F Y F C

murine VHR3    T R Q G L W F D S D G R G F D F W G Q G T T L T
human. VHaR3   A R Q G L W F D S D G R G F D F W G Q G S T V T

murine VHR3    V S S
human. VHaR3   V S S
```
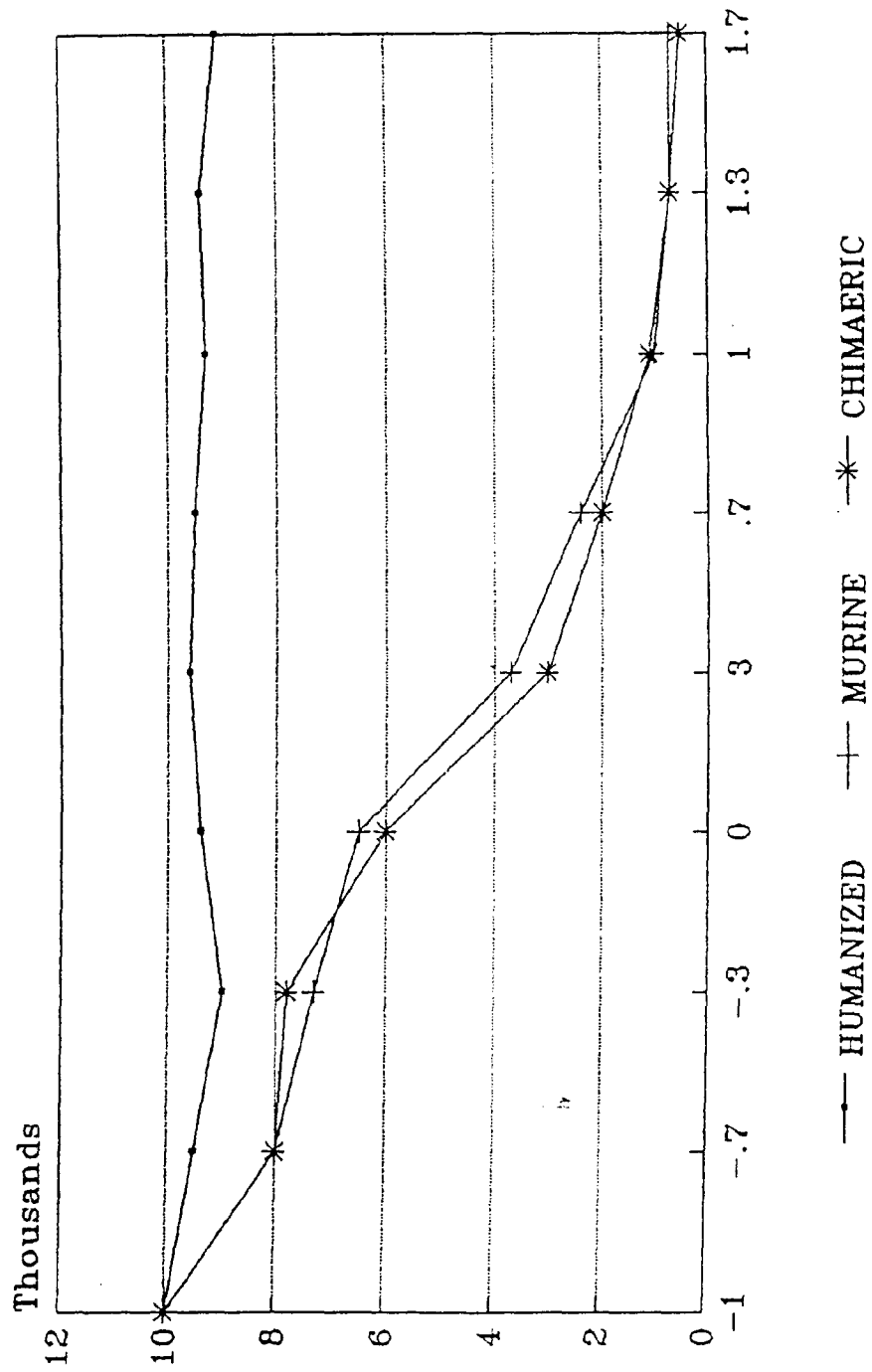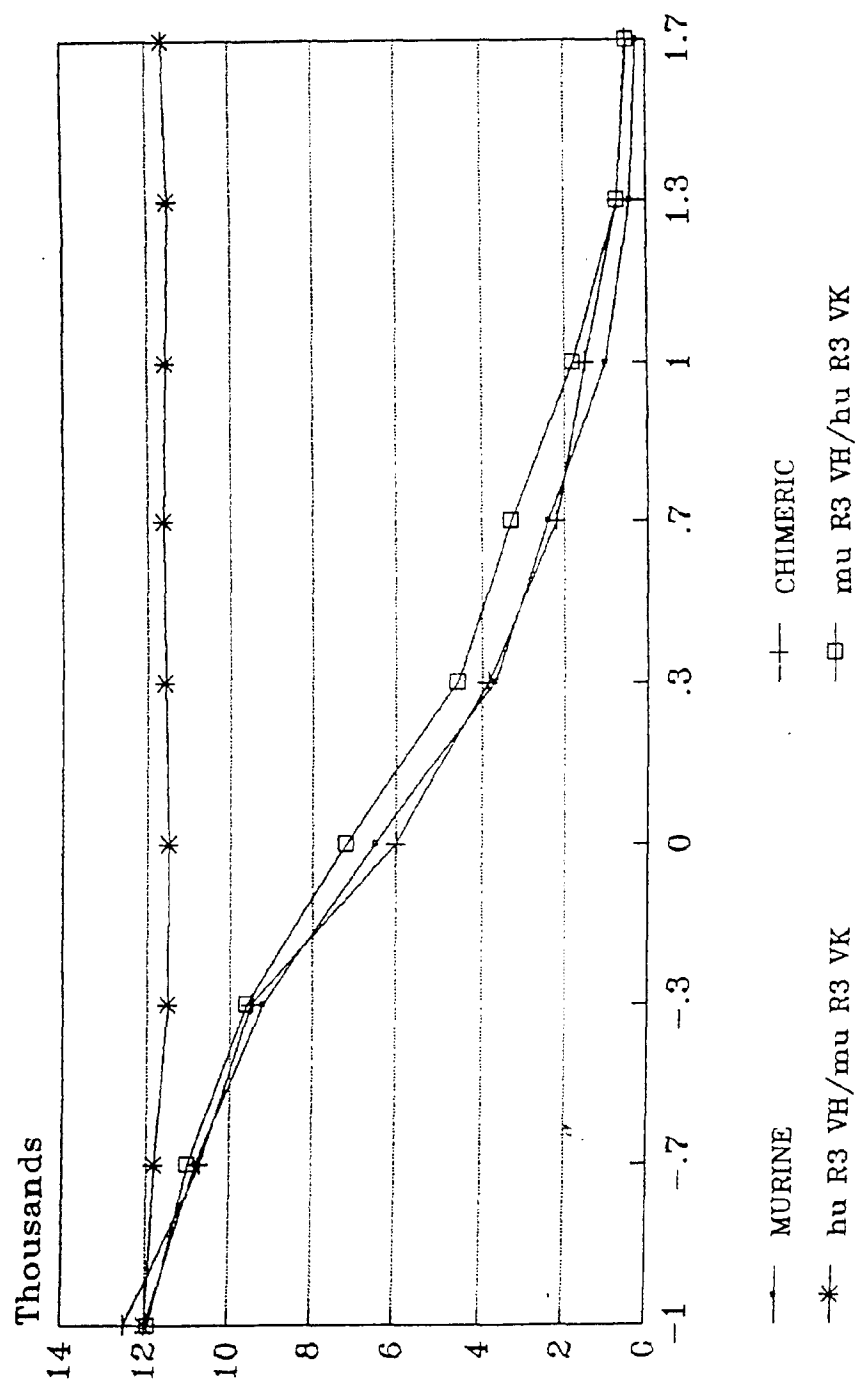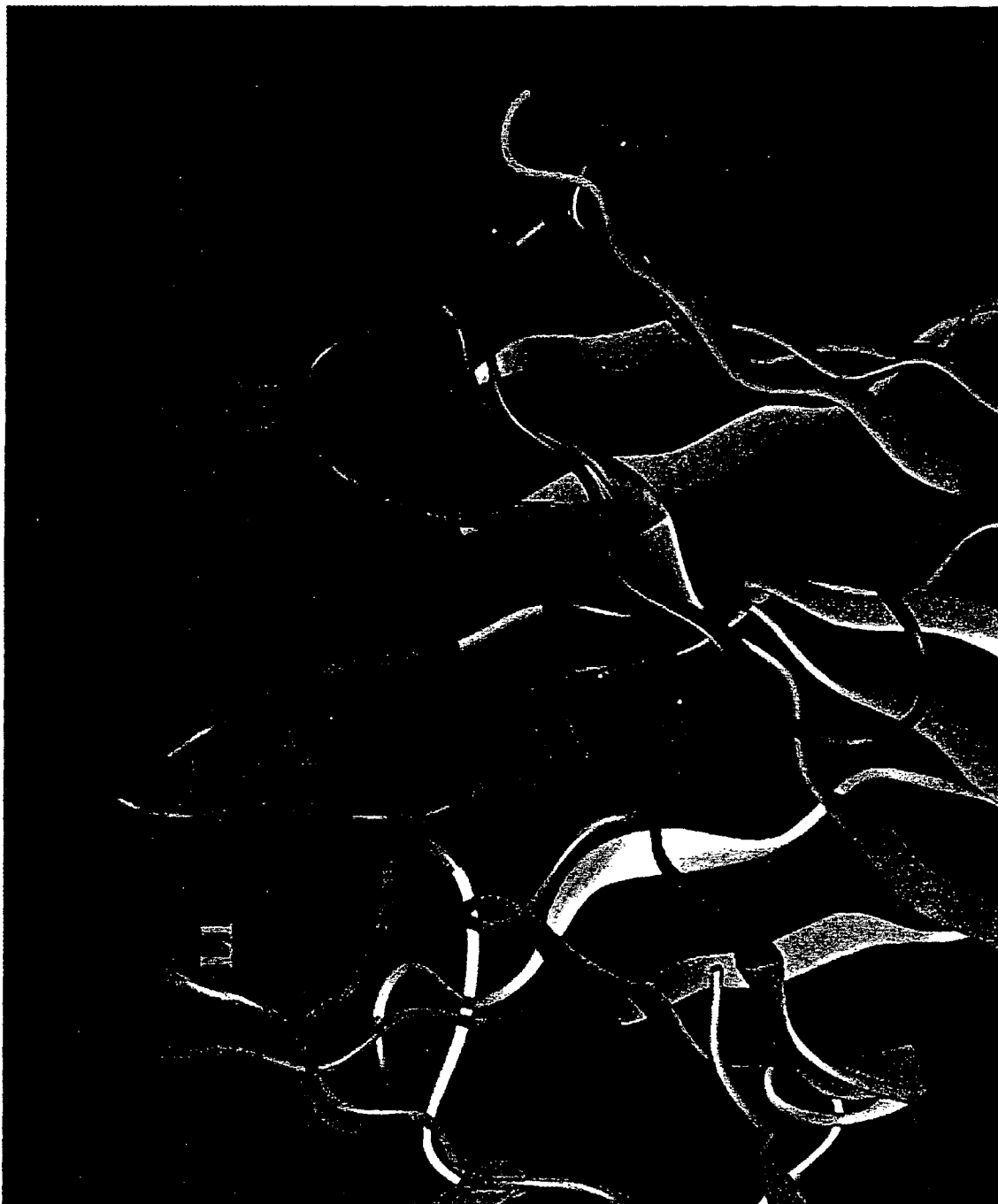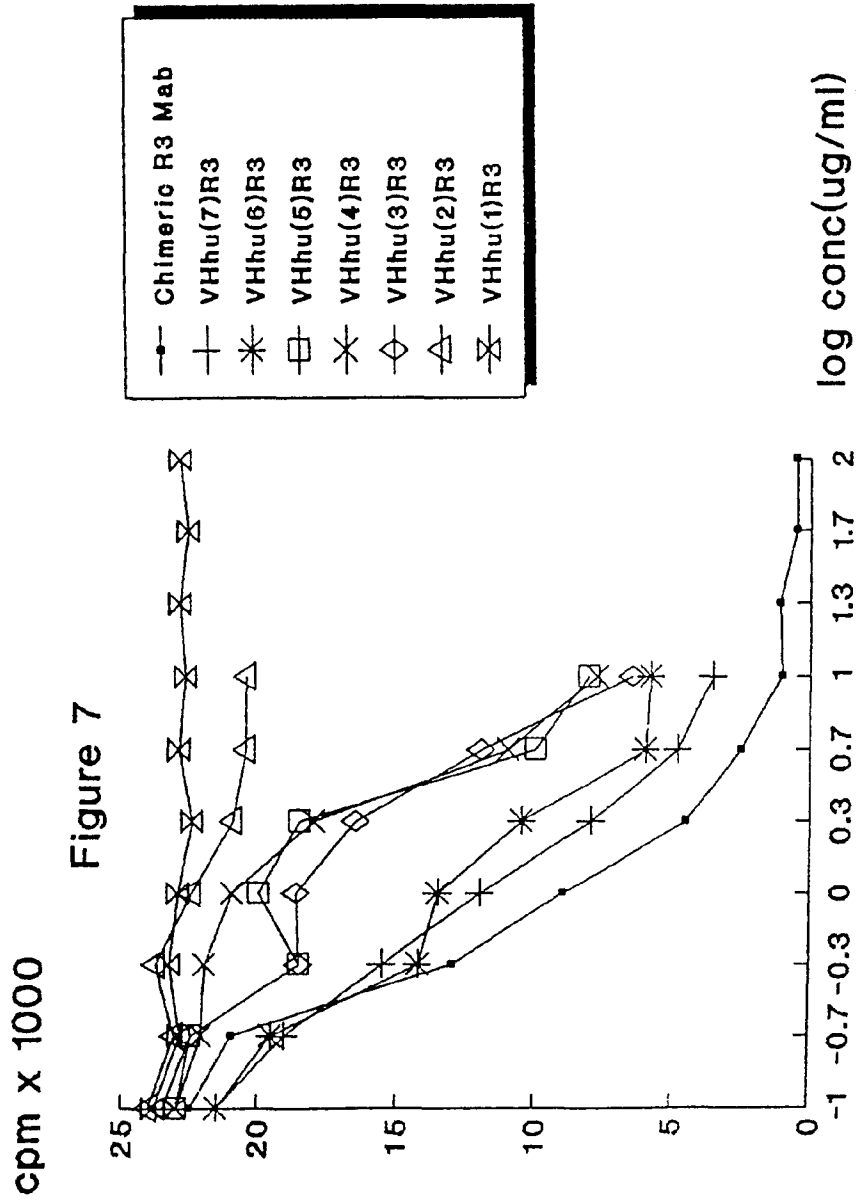
Figure 3

Figure 4

FIGURE 5: Comparison of the aminoacid sequences of reshaped humanized R3 heavy chain variable region. CDRs are underlined.

```
Hum.VH1R3    Q V Q L Q Q S G A E V K K P G S S V K V S C K A
Res.VH2R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH3R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH4R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH5R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH6R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH7R3    - - - - - - - - - - - - - - - - - - - - - - - -

Res.VH1R3    S G Y T F T N Y Y I Y W V R Q A P G Q G L E W I
Res.VH2R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH3R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH4R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH5R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH6R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH7R3    - - - - - - - - - - - - - - - - - - - - - - - -

Res.VH1R3    G G I N P T S G G S N F N E K F K T R V T I T V
Res.VH2R3    - - - - - - - - - - - - - - - - K A - - - -
Res.VH3R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH4R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH5R3    - - - - - - - - - - - - - - - - K A - - - -
Res.VH6R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH7R3    - - - - - - - - - - - - - - - - K A - - - -

Res.VH1R3    D E S T N T A Y M E L S S L R S E D T A F Y F C
Res.VH2R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH3R3    - - - S T - - - - - - - - - - - - - - - - - - -
Res.VH4R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH5R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH6R3    - - - S T - - - - - - - - - - - - - - - - - - -
Res.VH7R3    - - - S T - - - - - - - - - - - - - - - - - - -

Res.VH1R3    A R Q G L W F D S D G R G F D F W G Q G S T V T
Res.VH2R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH3R3    - - - - - - - - - - - - - - - - - - - - - - - -
Res.VH4R3    T - - - - - - - - - - - - - - - - - - - - - - -
Res.VH5R3    T - - - - - - - - - - - - - - - - - - - - - - -
Res.VH6R3    T - - - - - - - - - - - - - - - - - - - - - - -
Res.VH7R3    T - - - - - - - - - - - - - - - - - - - - - - -

Res.VH1R3    V S S
Res.VH2R3    - - -
Res.VH3R3    - - -
Res.VH4R3    - - -
Res.VH5R3    - - -
Res.VH6R3    - - -
Res.VH7R3    - - -
```

Figure 7

An examination of the effects of different
reshaped human Vh regions on antigen binding.

Figure 8